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Review

Smart Nucleic Acids as Future Therapeutics

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Nucleic acid therapeutics (NATs) hold promise in treating undruggable diseases and are recognized as the third major category of therapeutics in addition to small molecules and antibodies. Despite the milestones that NATs have made in clinical translation over the past decade, one important challenge pertains to increasing the specificity of this class of drugs. Activating NATs exclusively in disease-causing cells is highly desirable because it will safely broaden the application of NATs to a wider range of clinical indications. Smart NATs are triggered through a photo-uncaging reaction or a specific molecular input such as a transcript, protein, or small molecule, thus complementing the current strategy of targeting cells and tissues with receptor-specific ligands to enhance specificity. This review summarizes the programmable modalities that have been incorporated into NATs to build in responsive behaviors. We discuss the various inputs, transduction mechanisms, and output response functions that have been demonstrated to date.

Highlights

Smart nucleic acid therapeutics are a new class of drugs that detect molecular markers in a cell and respond by switching on/off and altering gene expression.

One example is the family of triggerable CRISPR technologies, which provide an efficient mechanism to control gene expression *in vitro* and *in vivo*.

Photochemical control provides spatiotemporal precision in triggering nucleic acid drugs, and the advent of photoregulation of mRNA translation was recently achieved *in vitro*.

Ribodevices can function as molecular switches to control the activation of mRNAs, guide RNAs, oligonucleotide therapeutics, and DNA nanostructures, either reversibly by using riboswitches or irreversibly using aptazymes.

Simple hybridization and strand-displacement reactions are highly programmable and provide a mechanism to create smart nucleic acid drugs that minimize off-target effects.

Opportunities of Nucleic Acid Therapeutics and the Challenges of Precision and Specificity

The primary role of nucleic acids *in vivo* is to encode and transduce genetic information. Interestingly, nucleic acids are also promising therapeutics that can modulate the expression of specific genes, and thus offer an alternative class of drugs to small molecules and protein-based therapeutics. Owing to their functional versatility, NATs can be designed to target each level of the central dogma. At the genome level, **guide RNAs** (gRNAs; see Glossary) in **CRISPR/Cas systems** and **triplex-forming nucleic acids** mediate genome editing. At the transcript level, mRNA and oligonucleotide RNA therapeutics {e.g., **antisense oligonucleotides (ASOs)**, small interfering RNAs (siRNAs), **microRNAs** (miRNAs), anti-miRNA, **DNAzymes** [1–3], and splicing-switching oligonucleotides} modulate the stability, abundance, or splicing of transcripts. At the protein level, **aptamers**, **DNA decoys**, and immune-stimulating oligonucleotides inhibit or stimulate protein activities. In addition, DNA nanostructures have also been developed as vehicles to deliver therapeutic payloads [4].

The discovery and development process of NATs is simple, fast, and cost-effective owing to high predictability of nucleic acid binding and structure. Over the past two decades, advances in chemical modification of the phosphodiester backbone, nucleobases, and ribose sugars [5], together with innovations in delivery formulations such as lipid nanoparticles (LNPs) [6], have resulted in NATs with high potency, low immunogenicity, and easy cell entry. Together this has led to tremendous progress toward clinical translation. Indeed, over the past 7 years, six ASOs and three siRNAs were approved by the FDA to address unmet medical needs by treating diseases lacking adequate treatment options, such as spinal muscular atrophy [7] and familial hypercholesterolemia [8]. Another clinical application of NATs pertains to the rapid development of vaccines. For example, Pfizer (https://clinicaltrials.gov/ct2/show/NCT04368728) and Moderna (https://clinicaltrials.gov/ct2/show/NCT04283461) are leading efforts using *in vitro* transcribed (IVT) mRNA to generate vaccines against SARS-CoV-2, the virus responsible for the COVID-19 pandemic [9,10]. The advantage of IVT mRNA is that it bears no risk of insertional mutagenesis,

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in contrast to DNA. Another exciting frontier for NATs is in genome editing, and early-stage clinical trials of CRISPR/Cas9 editing are showing evidence of safety and efficacy for *ex vivo* editing [11], with the first *in vivo* trial for treating Leber congenital amaurosis using EDIT-101, an adeno-associated virus type 5 (AAV5) vector expressing both Cas9 and gRNAs, [12] just launched.

Despite the milestones that NATs have made toward clinical translation, getting the therapeutics into the right cell type within the right tissue is a major roadblock to NATs for treating a wider range of clinical indications. Thus far, most NATs that have been approved or are under clinical testing are either directed to the liver by systemic delivery leveraging passive accumulation (siRNA-encapsulated LNPs, such as patisiran) [13] or ligand-mediated accumulation [*N*-acetylgalactosamine (GalNAc)–siRNA, such as givosiran] [14], or delivered locally (intrathecal injection for nusinersen, subretinal injection for EDIT-101). The development of innovative delivery technologies that enable efficient targeting of additional tissues and cell types is highly desirable and is the subject of multiple investigations that are nascent or in preclinical stages, such as LNPs that enable lung-targeted delivery [15].

An alternative strategy to enhance the specificity of NATs is through incorporating on/off switches into NATs, aiming to activate or deactivate NATs with specific exogenous or endogenous triggers. Creating these smart NATs involves combining programmable modalities within functional NATs. The trigger molecules are the 'input', the programmable modalities are signal 'transducers', and the output NAT functions are the 'effector' (Figure 1A). Smart NATs can be categorized based on the source of the input, the transducer, and the function of the effector (Figure 1B).

The present review focuses on recent progress in controlling NAT activity to achieve precise and specific therapeutics. We classify smart NATs broadly based on the mode of action of the transducer (programmable modality), including photochemical reactions, ribodevices, strand hybridization and displacement reactions, and protein-ligand binding.

Photochemical Control Provides Spatiotemporal Precision

As a bio-orthogonal trigger, light-induced regulation of NAT activity represents a very precise means of achieving high-resolution control in both a spatial and temporal fashion. Typically, photochemical control of the activity of oligonucleotide therapeutics is achieved through the introduction of photo-caged nucleobases or photolabile linkers. Activation or deactivation of ASOs [16–18], anti-miRNA [19], DNAzymes [17,20], transcription factor DNA decoys [21,22], triplex-forming oligonucleotides [23], and gRNAs [24,25] by light irradiation in mammalian cells or living model organisms has been achieved by incorporating photo-caged nucleobases or photolabile linkers into either the NATs themselves or their blocker sequences. As an example, Liu and coworkers developed a gRNA caged on the protospacer adjacent motif (PAM)-distal region, which is required for Cas9-mediated cleavage. This gRNA can bind to its target DNA but cannot induce cleavage until stimulated by light [26]. Another mechanism of light-activated NATs utilizes photochemical (λ = 350 nm) depurination of nucleobase mimics, which removes the purine bases and inhibits their binding function. For example, Struntz and coworkers demonstrated release of NF-κB transcription factor from its DNA decoy after light-induced depurination [27]. These methods rely on cleavage of chemical bonds and are hence irreversible. Reversible photoregulation of oligonucleotide function typically involves isomerization of a molecular photoswitch, such as azobenzene. The azobenzene group can be incorporated into oligonucleotides by insertion into the phosphate backbone as a linker or by tethering onto the oligonucleotides as a pendant group. Utilizing azobenzene as a linker, UV irradiation induces distortion of the phosphate backbone and can deactivate siRNA [28]. Another application of azobenzene

Glossary

Antisense oligonucleotides (ASOs): oligonucleotides complementary to target mRNA sequences that bind to the mRNA and recruit RNase H to cleave the mRNA.
Aptamers: oligonucleotides that bind to specific target molecules.
Aptazymes: engineered aptamers that self-cleave once bound to their ligand.
Capture-SELEX: a variant of the SELEX procedure in which DNA or RNA pools instead of the target are immobilized via a conserved docking sequence hybridized to the capture strand on the solid support.
Cell-SELEX: an *in vitro* selection method to generate aptamers that specifically bind to a cell type of interest.
CRISPR/Cas system: the clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein (Cas) system – a two-component (gRNA and Cas nuclease) gene-editing system.
Dicer: an endoribonuclease that cleaves double-stranded RNA and pre-miRNA into short siRNA and mature miRNA.
DNA decoy: synthetic DNA with high binding affinity for a transcription factor.
DNA origami: nanoscale 2D or 3D structures that are folded from DNA.
DNAzymes: synthetic DNA oligonucleotides composed of a catalytic core flanked by two binding arms which bind to and cleave a target RNA.
Drosha: an endoribonuclease that processes pri-miRNA into pre-miRNA in the nucleus.
Guide RNAs (gRNAs): synthetic RNAs composed of a scaffold region for Cas-binding and a spacer region for target sequence binding.
MicroRNAs (miRNAs): small RNAs with a length of ~22 nt which bind to the 3′-UTRs of mRNAs to cause their degradation or translation repression.
Pri-miRNA: a long RNA precursor containing a hairpin that can be processed by Drosha into pre-miRNA and then processed by Dicer into mature miRNA.
Ribosome binding site (RBS): a sequence of nucleotides upstream of the start codon of mRNA, which recruits ribosome to initiate protein translation.
Riboswitch: a regulatory fragment of an mRNA that binds to a small molecule and undergoes a conformational change to regulate protein translation.

backbone linkers is to control binding to complementary RNA. For example, Wu and colleagues introduced azobenzenes between ASOs and flanking inhibitory oligonucleotides to create light-activated ASOs [29]. Kamiya and colleagues used a similar strategy to generate an inducible DNAzyme by linking a complementary sequence with azobenzene to its catalytic core [30]. When azobenzene is tethered as a pendant group, UV irradiation stabilizes the *cis* conformation, which is nonplanar and causes steric hindrance and thus dissociation of a duplex. Leveraging this strategy, photoregulation of the RNA-cleavage rate of DNAzymes [31] and ASOs [32] was controlled by alternately applying UV and visible light.

Utilizing photolabile linkers, light-sensitive DNA nanostructures were also designed, including a hollow nanocuboid for light-triggered release of cargo protein [33], and a **DNA origami** sphere that can transform into two tethered hemispheres by light irradiation [34]. Triggered by UV or visible light irradiation, hybridization and dehybridization of two oligonucleotides with azobenezene linkers enable a bipyramidal DNA nanocapsule to open and close reversibly [35,36].

Controlling mRNA translation by light can be achieved by a combination of light-triggered uncaging or isomerization of small-molecule ligands with riboregulatory devices in the untranslated region of the mRNA, which is called a photo-**riboswitch**. Examples include **aptazymes** that are activated to self-cleave by binding to uncaged ligands (e.g., guanine and glucosamine-6-phosphate, GlcN6P) [37,38], and a riboswitch that selectively binds to the *trans* isoform of an amino stiff-stilbene [39]. In addition to photo-riboswitches, a recent study reported a unique method for photoregulating mRNA translation mediated by light-triggered protein–protein interaction. In this work, mRNA is tagged with binding sites for bacteriophage MS2 coat protein, which allows labeling with cryptochrome 2 (CRY2) and crosslinking of mRNAs with cryptochrome-interacting basic-helix–loop–helix 1 (CIB1)-fused multimeric proteins in a blue light-specific manner, resulting in translation repression of the mRNA because of inaccessibility for ribosome binding [40]. Although this platform is not suitable for controlling mRNA therapeutics because of temporary translation inhibition and the complexity of engineering fused proteins, it provides a new direction for deactivating and activating mRNA translation by clustering and disassembly.

Photochemical control provides spatiotemporal precision, but tissue penetration of light is a major hurdle for activating NATs in deep tissues. To solve this issue, different groups have worked on designing BODIPY [41] or cyanine [42,43] derivative linkers or two photon-sensitive linkers [22,44] that can be cleaved by visible or near-IR light to push the penetration limit.

Aptamer-Based Ribodevices Enable Small Molecule- or Protein-Triggered Activation or Deactivation

Riboswitches are naturally occurring RNA motifs which incorporate an aptamer (Box 1) as well as a gene-regulation domain. When the aptamer binds to its small-molecule or protein target, this leads to a conformational switch in the RNA that then triggers or inhibits gene expression kinetically or thermodynamically. Riboswitches, which are most commonly found in bacteria, have inspired several technologies for gene regulation. This is typically achieved by engineering RNA aptamer-based ribodevices, including riboswitches and aptazymes, into the non-coding regions of mRNA so as to control its splicing, translation, and degradation [45]. The most common examples of this approach involve inserting a riboswitch into the **5′-untranslated region** (5′-UTR), which undergoes a ligand binding-induced structural change that exposures a blocked **ribosome binding site** (RBS) (Figure 2A). Hence, ligand–aptamer binding enables modulation of translation. Another class of engineered ribodevices involves allosteric self-cleaving ribozymes imbedded within the mRNA. Ligand-binding triggers self-cleavage of the

RNA-induced silencing complex (RISC): a multiprotein complex that incorporates a guide strand of siRNA or miRNA to guide its binding and cleavage of target mRNA.
RNase H: a sequence-independent endonuclease that selectively cleaves RNA in an RNA/DNA duplex.
SELEX: systemic evolution of ligands by exponential enrichment – an *in vitro* selection method to discover aptamers that specifically bind to a target molecule.
Toehold-mediated displacement reaction: a reaction between a single-stranded DNA or RNA and a double-stranded DNA or RNA with a toehold to displace or exchange one strand with another.
Triplex-forming nucleic acid: oligonucleotides that can form a triplex with DNA or RNA through Hoogsteen base-pairing.
3′-Untranslated region (3′-UTR): an untranslated region of mRNA that follows the translation termination codon and contains regulatory regions (e.g., miRNA target site) that regulate mRNA stability and mRNA translation.
5′-Untranslated region (5′-UTR): an untranslated region of mRNA that is directly upstream of the start codon and which regulates the translation of the mRNA.

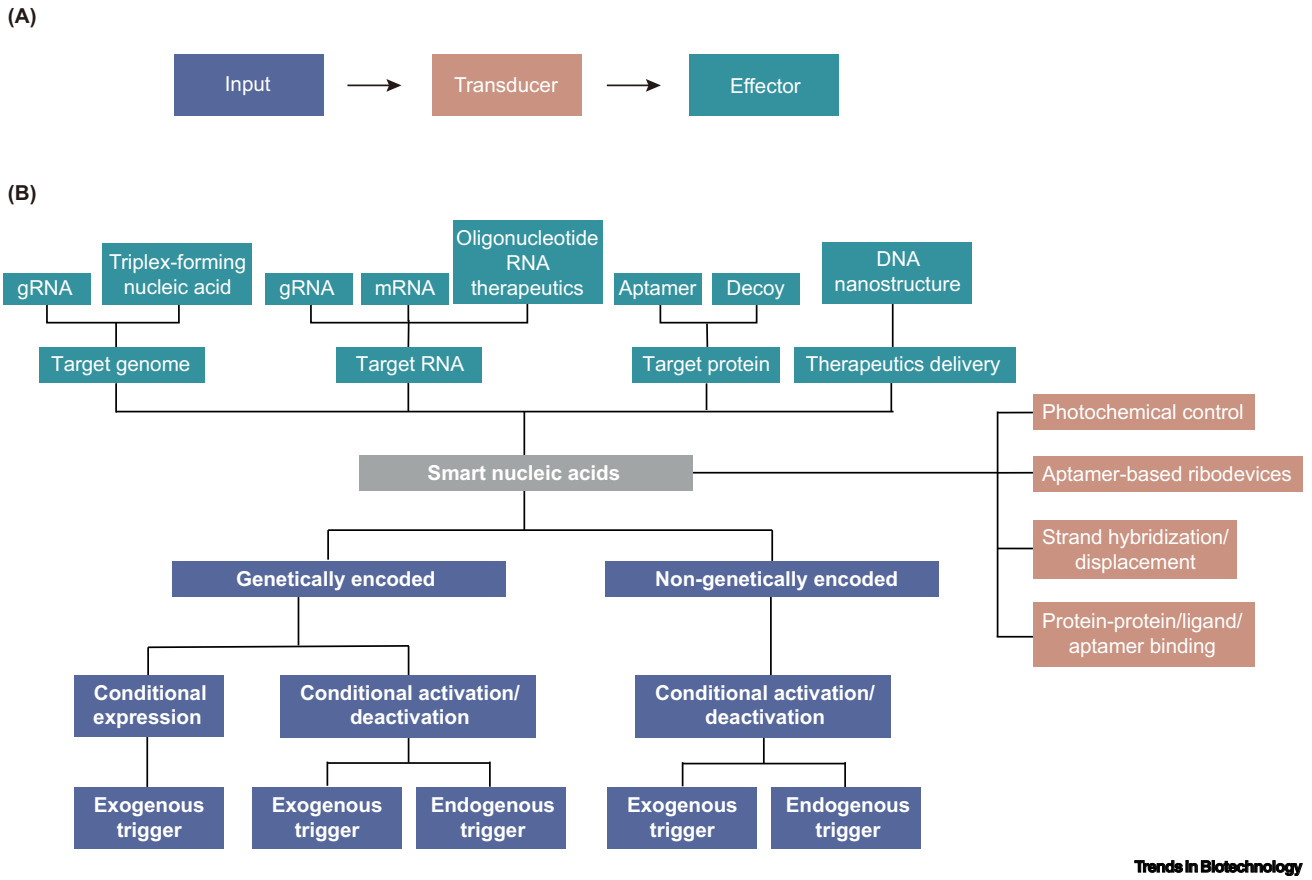


Figure 1. Schematic Showing the General Components and Different Types of Smart Nucleic Acid Therapeutics (NATs). (A) Smart NATs are comprised of three main components: the trigger molecule is the 'input' (blue), the 'transducer' is typically a programmable conformational or structural change (brown), and the output function is the 'effector' (turquoise). (B) Smart NATs can be categorized based on the source of the input (genetically encoded, non-genetically encoded), the transducer (photochemical control, ribodevices, hybridization/strand displacement, protein–protein/ligand/aptamer binding), and the function of the effector (genome editing, RNA regulation, protein binding, therapeutics delivery). Abbreviation: gRNA, guide RNA.

mRNA and translation repression [46,47] (Figure 2B). More comprehensive discussion of natural and engineered ribodevices can be found in other review articles [48–50] and is not the focus of our review.

mRNA stability and translation are also naturally controlled by the expression of miRNAs which bind to the **3′-untranslated region** (3′-UTR) of the target mRNA, causing translation repression [51]. Ribodevices can be used to tune gene expression by interfering with miRNA functions. For example, Farzan and colleagues created a riboswitch engineered into the 3′-UTR of an mRNA to conceal the miRNA target sequence upon ligand binding and thus rescue protein expression [52] (Figure 2C). To the best of our knowledge, these ribodevice-controlled gene regulation systems have only been introduced into mammalian cells using plasmids or viral vectors, and they have yet to be delivered using IVT mRNA. We anticipate that the next generation of *in vivo* ribodevices will be delivered using mRNA akin to the advent of mRNA vaccines and protein replacement therapies.

Riboswitches and aptazymes have also been engineered into the 5′ or 3′ ends of gRNAs to create conditional CRISPR endonuclease activity. Consider, for example, an extended gRNA with an

Box 1. Aptamers, SELEX, and Structure-Switching Aptamers

Aptamers are single-stranded nucleic acids that selectively bind to target molecules such as small molecules, peptides and proteins. Aptamers naturally exist in riboswitches but can also be generated through a process called 'systematic evolution of ligands via exponential enrichment' (SELEX), as shown in Figure IA [108]. A library of synthetic oligonucleotides with random sequences flanked by constant regions are exposed to the target ligand. The unbound sequences are removed, whereas the sequences bound to the targets are released and amplified by PCR for subsequent rounds of binding. After several rounds of selection and amplification, sequences with high affinity for the target ligand are enriched. These sequences are then sequenced, and their binding affinity and specificity are evaluated. When live cells are used as the target ligand, aptamers that bind to specific cell types can be identified through a similar iterative process called Cell-SELEX [98].

Structure-switching aptamers are aptamers that undergo a conformational change upon target binding [99,109]. The conformational change can subsequently be transduced into an output signal. Structure-switching aptamers can be created through engineering existing aptamers to introduce structure-switching functionality [110]. However, the process can be challenging and time-consuming because an optimal thermodynamic balance between the aptamer–target complex and the aptamer–nucleic acid complex requires iterative sequence design and experimentation [111]. Another strategy to generate structure-switching aptamers is to directly select aptamers with inherent structure-switching capability through structure-switching SELEX (Figure IB) or Capture-SELEX (Figure IC). In these methods, the random sequence library is hybridized to a docking strand on magnetic beads, and only the sequences that dissociate from the beads upon target exposure are enriched. In structure-switching SELEX, the primer-binding site is hybridized to the docking stand; it therefore only works when the primer-binding sequence takes part in target binding [112]. By contrast, Capture-SELEX is more general because the docking sequence is complementary to the random region of the synthetic library [113]. Capture-SELEX has been performed to generate a paromomycin-binding synthetic riboswitch, as reported by Boussebayle and coworkers [61].

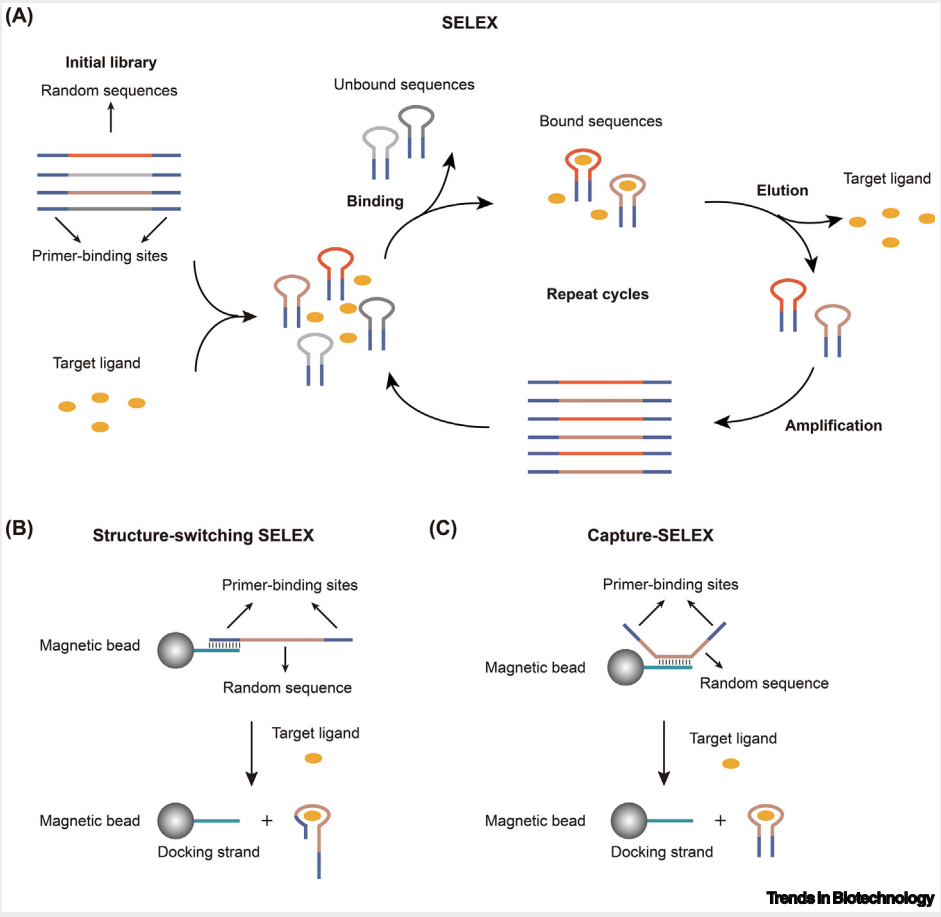
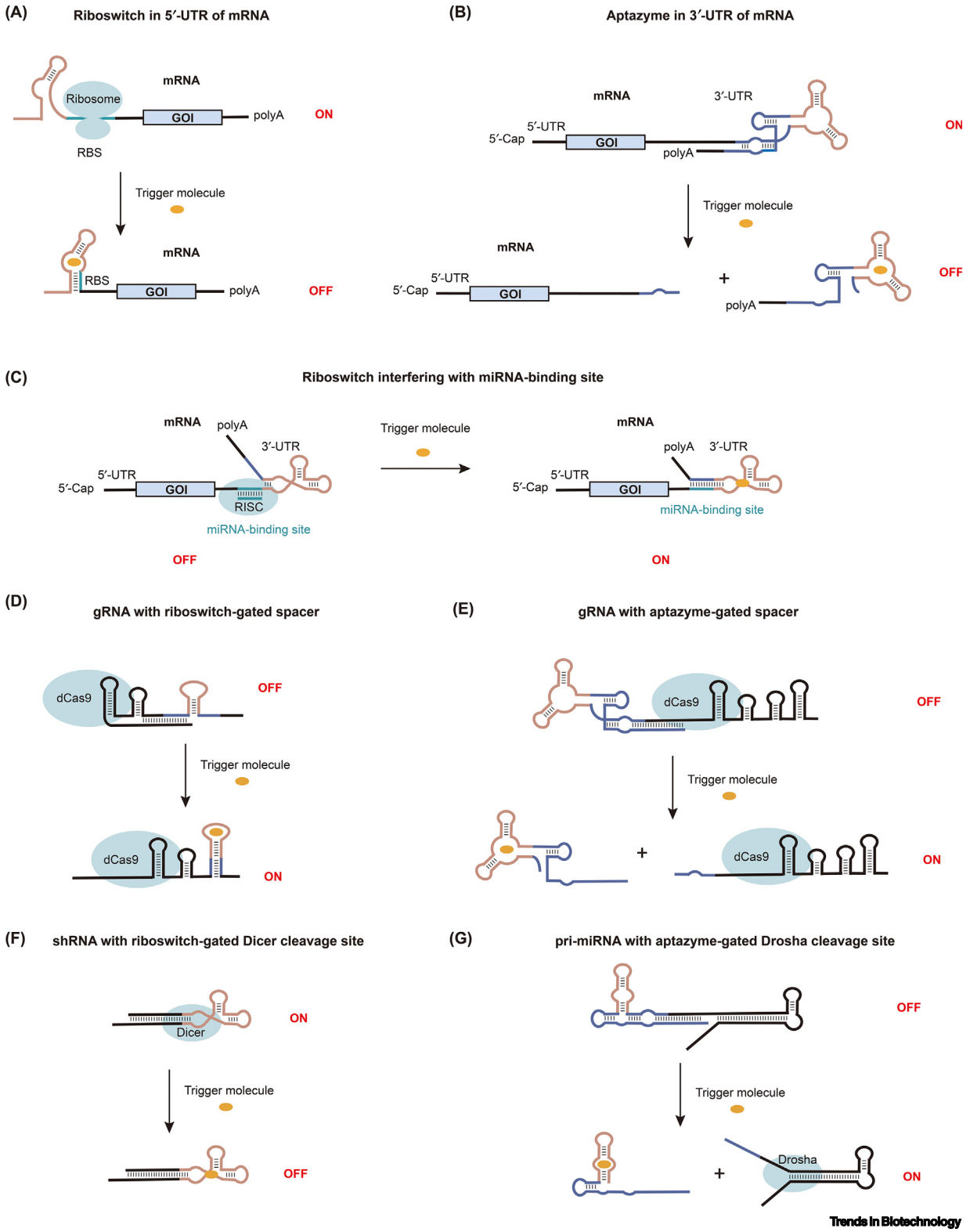


Figure I. SELEX, Structure-Switching SELEX, and Capture-SELEX. (A) Conventional SELEX to generate aptamers that bind to specific target molecules. (B) Structure-switching SELEX, and (C) Capture-SELEX directly select



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aptamer at its 3′ end (Figure 2D) [53]. In the absence of the ligand molecule, the spacer region of the gRNA is paired with its complementary sequence in the 3′ extension of the gRNA, and therefore cannot bind to its target DNA; however, when a ligand binds to the aptamer it induces a conformational change that allows the spacer region to interact with the corresponding DNA sequence. By extending the gRNA with a variety of aptamers that recognize small molecules or proteins, deactivation or activation of the transcription of the gene of interest in response to these triggers was achieved by coexpression of dCas9 or dCas9 fused to the herpesvirus transcriptional activator VP64 in mammalian cells [53]. This signal conductor design was further applied to redirect oncogenic signaling to the antioncogenic pathway [53]. Small molecule-triggered restoration of gRNA activity was also achieved by inserting an aptazyme that contains a theophylline or guanine-binding aptamer between the spacer region and a 5′-extended blocking sequence, which transduces the presence of a small molecule into ribozyme self-cleavage. The addition of ligands triggers self-cleavage of the aptazyme and removal of the blocking sequence from the gRNA, thus enabling genome editing, base editing, and transcription activation (Figure 2E) [54]. Self-cleavage and degradation of gRNA with an aptazyme inserted into a different region (e.g., the stem-loop region) of the gRNA backbone was also designed and implemented by Chen and colleagues [55].

In addition to mRNA and gRNAs, ribodevices have also been integrated into siRNA or miRNA precursors to enable small molecule-triggered/inhibited RNA interference. As an example, incorporation of a theophylline aptamer into the loop region or basal segment of a short hairpin RNA (shRNA) (Figure 2F) or a **pri-miRNA** led to dose-dependent inhibition of RNAi by theophylline because cleavage by **Dicer** or **Drosha** was inhibited [56,57]. In another example, a pri-miRNA analog was extended with a theophylline aptazyme and an inhibition strand that hybridized to the 5′ end of the pri-miRNA analog, thus preventing its processing by Drosha. Addition of theophylline results in self-cleavage and the generation of a proper Drosha substrate, which can then be processed sequentially by Drosha and Dicer to generate mature siRNA (Figure 2G) [58]. A good example of using these ribodevices for therapeutic applications was recently published by Lee and colleagues, where the ribozyme detects hepatitis C virus (HCV) nonstructural protein 5B (NS5B) and responds by activating anti-miR-122 function in liver cells, thus providing a triggerable therapeutic for HCV infection [59].

Riboswitches can also be incorporated into dynamic DNA nanostructures for small molecule-triggered cargo release. For example, Banerjee and coworkers reported an icosahedral nanostructure stabilized with a structure-switching cyclic-di-GMP (cdGMP) aptamer. Upon exposure to cdGMP, the aptamer was remodeled, resulting in dissociation of the icosahedron and release of the encapsulated cargo [60].

Figure 2. Ribodevices Enable Small Molecule- or Protein-Triggered Activation or Deactivation of mRNA (A–C), Guide RNA (gRNA) (D,E), and siRNA or miRNA (F,G). (A) A riboswitch in the 5′-untranslated region (5′-UTR) of the mRNA blocks the ribosome binding site (RBS) and represses expression of the gene of interest (GOI) upon trigger-molecule binding. Image adapted, with permission, from [116]. (B) An aptazyme in the 3′-UTR of the mRNA self-cleaves and causes degradation of the mRNA upon-trigger molecule binding. Image adapted, with permission, from [46]. (C) A riboswitch in the 3′-UTR of mRNA conceals an miRNA-binding site upon trigger-molecule binding, hence stabilizing the mRNA and switching on gene expression. Image adapted, with permission, from [52]. (D) A gRNA designed with its spacer region hybridized to an extended sequence containing a riboswitch that keeps the Cas9 in an inactive state until the trigger molecule induces a conformational change of the aptamer leading to exposure of the spacer domain. Image adapted, with permission, from [53]. (E) A gRNA designed with a 5′ extension containing an aptazyme and a complementary sequence of the spacer region. Ligand binding induces self-cleavage and removal of the extension, and thus exposes the spacer region. Image adapted, with permission, from [54]. (F) A short hairpin RNA (shRNA) with its loop domain replaced by a riboswitch. Ligand binding triggers a structural change in the aptamer and thus blocks the Dicer binding site and inhibits processing of the shRNA into siRNA. Image adapted, with permission, from [56]. (G) A pri-miRNA is extended with an aptazyme which blocks the Drosha cleavage site. Upon ligand binding, the aptazyme is cleaved and removed, leading to Drosha processing of the pri-miRNA. Image adapted, with permission, from [58].

Although ribodevices responding to several ligands have been constructed, extension of this platform to diverse inputs, especially disease-related molecules for autonomous therapy, remains a challenge. This is because of the limited number of ligands recognized by riboswitches with high affinity and specificity within a physiological concentration range and with necessary RNA conformational switching. To apply ribodevices as smart NATs with translational capability, new synthetic riboswitches that respond to endogenous disease markers need to be generated. This is typically time-consuming and can be accomplished using modified **SELEX** methods, such as structure-switching SELEX or **Capture-SELEX**, followed by intracellular screening [61] (Box 1).

Hybridization and Strand Displacement Enable Transcript/Synthetic Oligonucleotide Responsivity

Hybridization/Strand Displacement Only

Hybridization to occlude an essential region of a NAT by RNA or DNA is an effective strategy for abrogating its activity. Two good examples of this strategy are anti-miRNA and REVERSIR GalNAc-ASOs [62]. Furthermore, loop-mediated hybridization and the **toehold-mediated displacement reaction** (Box 2) [63] provide two possibilities to unblock nucleic acids, exposing the essential region for target binding and thus recovering activity.

Inspired by the innate input/output response of riboswitches, the field has engineered an array of riboregulatory devices that respond to cognate RNAs [64–67] to control mRNA translation. For example, in an engineered riboregulator developed by Isaacs and colleagues, the RBS of mRNA was concealed by a *cis*-repressing complementary sequence directly upstream of the RBS, which forms a stem-loop structure that interferes with ribosome binding. A small activating RNA unfolds the stem-loop by hybridizing to the loop and the complementary sequence, thus exposing the RBS and permitting translation (Figure 3A) [68]. An improvement to this design was demonstrated by Green and colleagues, where the RBS domain was encoded into the hairpin loop of the toehold switch. The trigger can therefore use virtually any sequence and is not limited to incorporating the RBS sequence itself (Figure 3B) [69]. The use of toehold-mediated displacement reactions, as opposed to loop-mediated hybridization, enables stronger thermodynamics and improved kinetics, resulting in a wider dynamic range for the response function. Because the toehold switch design can accept trigger RNAs with arbitrary sequences, these elements can be activated by endogenous RNAs for autonomous control of gene expression. Toehold switches controlled by endogenous miRNA were demonstrated in mammalian cells by Wang and coworkers [70], indicating the potential application of this strategy in autonomous mRNA therapeutics. Furthermore, Kim and colleagues designed an ultrasensitive toehold switch composed of multiple hairpins sensing the same RNA trigger, resulting in an increased apparent Hill coefficient with decreasing hairpin-to-hairpin spacing or increasing hairpin number [71]. In addition to 'off-to-on' toehold switches, translational repressors were recently designed as variations of 'on-to-off' toehold switches. For example, toehold repressors that turn off translation through RNA-triggered disruption of a hairpin upstream of an exposed RBS were developed recently (Figure 3C) [72]. Another type of 'on-to-off' toehold switch is a three-way junction repressor, in which an unstable hairpin containing the RBS and start codon allows translation to proceed. However, when a trigger RNA binds to the two domains flanking the hairpin, the hairpin becomes sufficiently stable to block translation (Figure 3D) [72]. These methods provide a convenient approach to control gene expression by using innate expressed RNA inputs. Next, we describe more recent approaches that leverage the Cas9 nuclease system to achieve the same goal of controlling gene expression using RNA inputs.

The CRISPR/Cas9 system offers a very potent and effective approach that can leverage hybridization or displacement reactions to control gene expression. Akin to the toehold-displacement

Box 2. Toehold-Mediated Reactions

Toehold-mediated reactions are a key family of reactions that enable the responsivity of smart nucleic acid devices to nucleic acid inputs [63]. Figure IA illustrates an example of a toehold-mediated strand displacement reaction. A double-stranded DNA complex X is composed of two strands – a substrate strand A and an incumbent strand B. The substrate strand A has a single-stranded overhang region (α domain) that is the so-called 'toehold'. A third strand C, referred to as the invader strand, is complementary to strand A. The displacement reaction is initiated when the α′ domain on the invader strand C hybridizes to the toehold α domain on the substrate strand A. End-fraying of the incumbent strand B allows the invader strand C to bind to the substrate stand A with one more base pair. The invader strand C then competes with the incumbent strand B for binding to the β domain on the substrate stand A through a random-walk process. This competing process is called three-way branch migration. With completion of branch migration, the incumbent strand B is completely displaced, and the substrate strand A and invader strand C are fully hybridized into a new DNA duplex Y.

The displacement reaction is thermodynamically favored, but kinetically hindered. The initial toehold-binding step lowers the activation barrier and increases the rate of the reaction by increasing the attempt frequency of branch migration [114]. Because the substrate strand A lacks a toehold domain for the incumbent strand B to bind to, the rate constant for the reverse reaction going from duplex Y to complex X is diminishingly small. However, if the incumbent strand B possesses a γ′ domain that must spontaneously dissociate for the displacement reaction to complete, a new toehold γ is exposed while the original toehold α is sequestered, as shown in Figure IB. This process is called 'toehold exchange', which enables further toehold-mediated reactions [115].

Toehold-mediated reactions allow the exchange of the input (the invader) with the output therapeutic effector or inhibitor (the incumbent), thus resulting in reversible activation or deactivation of the smart therapeutics.

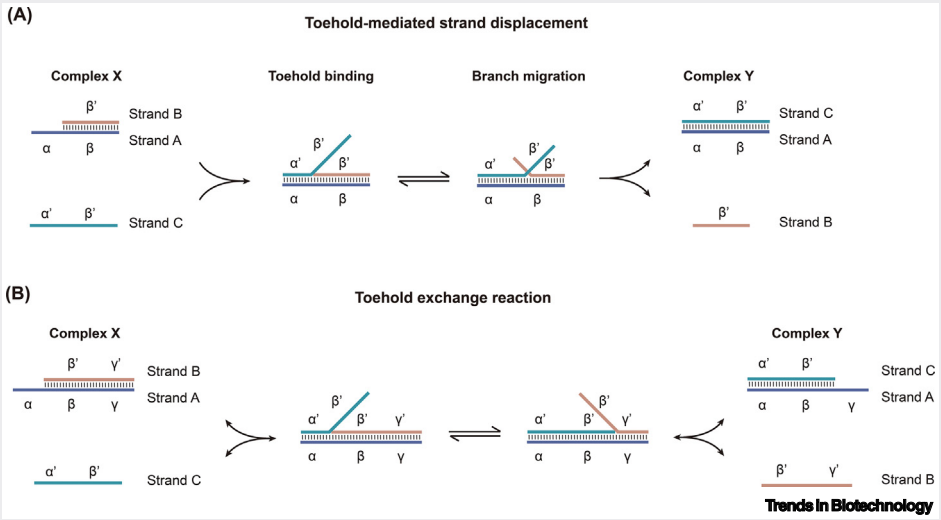

Figure I. Toehold-Mediated Reactions. (A) Toehold-mediated strand displacement reaction, and (B) toehold exchange reaction.

approaches described in the previous text, the gRNA can be deactivated or activated by binding to an input transcript (Figure 4A–C). The general strategy is to tune the level of repression of gRNA by designing antisense RNAs that conceal different regions of a gRNA [73]. For example, toehold-gated gRNA (thgRNA) is designed with a spacer domain hidden in a hairpin such that Cas9 activity is diminished. Once the thgRNA binds to an RNA trigger strand complementary to the toehold and branch-migration domains, it is activated and switches on Cas9 activity (Figure 4A) [74]. Another approach is to design the gRNA with a 3′ extension that blocks its scaffold domain and abolishes Cas9 binding; this gRNA recovers its function upon binding a transcript that induces a structural change (Figure 4B) [75]. One of the few examples using the more recently discovered Cas12a nuclease, rather than Cas9, allows Cas12a binding and activation when the stem-loop of the gRNA is unwound by the trigger RNA [76]. In addition to 'off-to-on'

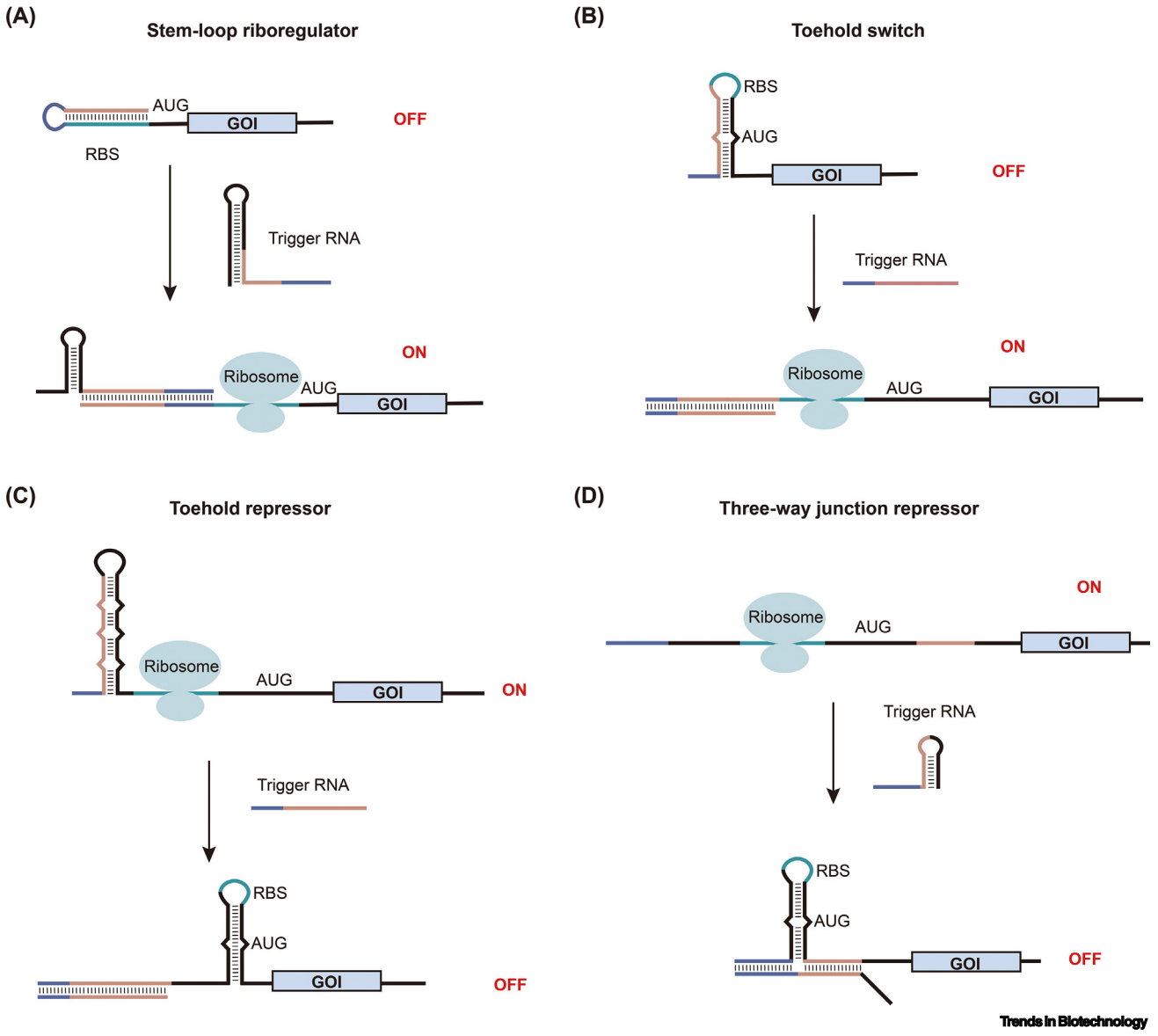

Figure 3. Hybridization and Strand Displacement Enable Transcript Responsivity of mRNA. (A) A stem-loop riboregulator interferes with ribosome binding and permits translation upon binding of the trigger RNA. Image adapted, with permission, from [68]. (B) An engineered toehold switch leverages a toehold-mediated displacement reaction to expose the RBS and trigger translation. Image adapted, with permission, from [69]. (C) A toehold repressor turns off translation upon trigger RNA binding. Here the RNA binds to the toehold switch and generates a new stem-loop that blocks the RBS and inhibits translation. Image adapted, with permission, from [72]. (D) A three-way junction repressor stabilizes an RBS-containing stem-loop in the presence of trigger RNA, and thus switches off translation. Image adapted, with permission, from [72]. Abbreviations: GOI, gene of interest; RBS, ribosome binding site.

gRNAs, a terminator switch enables an 'on-to-off' response in conditional gRNAs by modifying the terminator region of the gRNA. Hybridization of the modified terminator domain to the gRNA triggers a conformational change of the gRNA that inhibits dCas9 function (Figure 4C) [77]. These previously-mentioned systems were successfully demonstrated in *Escherichia coli* and/or mammalian cells, and afford dynamic gRNA tools for potential controlled or specific CRISPR applications. Given the need to better control off-target activity of the Cas9 system, it is likely that more sophisticated trigger mechanisms will be developed in the coming years.

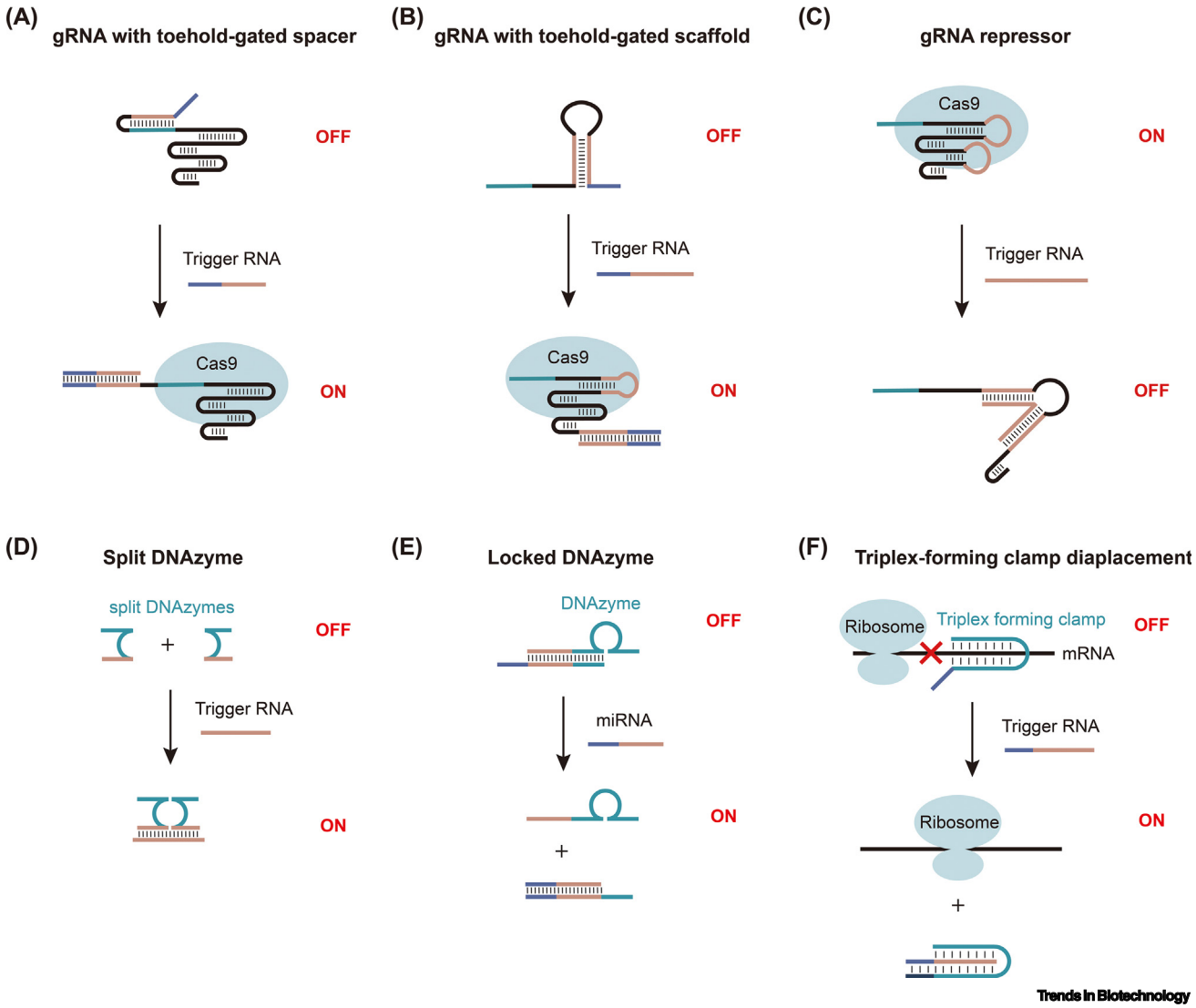

Figure 4. Hybridization and Strand Displacement Enable Transcript Responsivity of Guide RNA (gRNA; A–C) and Oligonucleotide RNA Therapeutics (D–F). (A) A gRNA with toehold-gated spacer domain is activated by a trigger RNA to expose the spacer domain. Image adapted, with permission, from [74]. (B) A gRNA with toehold-gated scaffold domain is activated to enable Cas9 protein binding. Image adapted, with permission, from [75]. (C) A gRNA with a modified stem-loop structure alters its shape upon trigger-RNA binding to disable Cas9 protein binding. Image adapted, with permission, from [77]. (D) Split DNAzymes are activated upon trigger-RNA binding-mediated assembly. Image adapted, with permission, from [78]. (E) A conditional DNAzyme with one binding arm locked is activated by miRNA through toehold exchange. Image adapted, with permission, from [79]. (F) A triplex-forming clamp dissociates from the target mRNA as a result of toehold-mediated displacement by the trigger RNA. Image adapted, with permission, from [80].

Strand-hybridization and -displacement reactions can also be incorporated into the design of conditional oligonucleotide therapeutics (siRNAs, ASOs, shRNAs, DNAzymes, etc.) (Figure 4D–F). For example, a split DNAzyme was designed for controlled assembly and activation of DNAzyme activity by pulling the split DNAzyme halves together through binding to an input strand (Figure 4D) [78]. Zhang and coworkers created a locked DNAzyme that is selectively switched on in response to endogenous miRNA inputs (Figure 4E) [79]. In this design, one binding arm of the DNAzyme was extended with a partial miRNA sequence, and the binding arm as well as the extension were blocked by hybridization to a lock strand that can bind to the miRNA input. The

miRNA input triggers dehybridization of the locked DNAzyme through toehold exchange (Box 2), thus activating the cleavage activity of the DNAzyme for its target mRNA. A final interesting example of using a strand-displacement reaction to trigger gene expression is the use of an inactivating triplex forming nucleic acid clamp that blocks the ribosome (Figure 4F) [80]. Here the input RNA binds to a toehold on the clamp and drives its displacement from the mRNA and thus rescuing mRNA translation activity.

Dynamic DNA nanostructures for cargo exposure or release were also designed leveraging hybridization or displacement reactions (Figure 5A–C). A cylindrical nanorobot with a switchable flap was developed which opens in response to target hybridization. In this case, the flap is linked to the chassis of the structure using single-stranded (ss) DNA, and once the ssDNA is hybridized it becomes shorter (due to forming the duplex), pulling the flap open (Figure 5A) [81]. The toehold-mediated strand displacement reaction has been used in multiple examples to achieve DNA-triggered release of cargo. For example, the lid of a DNA nanobox was opened with 'key' oligonucleotides (Figure 5B) [82], and a DNA nanosuitcase that encapsulates siRNA released its cargo upon displacement by the trigger strands [83]. To create a reversible dynamic system, Grossi and colleagues created a DNA vault that opens and closes by the addition of excess opening key and closing key strands (Figure 5C) [84]. These examples demonstrate the versatility of using toehold-displacement reactions to generate input/output functions that control drug release.

Hybridization- and Displacement-Mediated Enzyme Cleavage

Hybridization and strand displacement can generate enzyme binding sites for NATs, leading to their activation or destabilization. The advantage of this strategy is the potential for amplification of the original input signal. For example, mRNA therapeutics that are controlled by miRNA inputs were created by Jain and coworkers. In this case, the mRNA is engineered with miRNA targeting sites in its 3′-UTR, which allows enhanced degradation of the mRNA in cells expressing the specific miRNA (Figure 6A). The biomedical implications of this strategy were also demonstrated by expressing a toxic protein in cancer cells while silencing the mRNA in miR-122-expressing liver cells, which enabled more selective destruction of cancer cells with reduced hepatotoxicity [85]. Subsequently, Lee and colleagues leveraged this concept to achieve gene editing in the liver while repressing gene editing in unintended tissues. This was achieved by encoding anti-CRISPR mRNA that was selectively degraded by miR-122 that is abundantly expressed in the liver [86].

Mature gRNA can also be generated by a similar mechanism that utilizes miRNA-directed **RNA-induced silencing complex** (RISC) cleavage. An miRNA-mediated gRNA release platform was achieved by flanking the gRNA sequence with two miRNA binding sites (Figure 6B). The generation of the mature gRNA and its activity reflect the expression level of the miRNA in specific cell types. This platform can also be adapted to respond to siRNAs. Because many miRNAs and siRNAs are restricted to cells of specific lineages or at different development or disease stages, this platform may be exploited for cell type-specific CRISPR/Cas9 functions (Figure 6B) [87].

In addition to the RISC, **RNase H** is another endogenous enzyme that can be leveraged for hybridization-mediated activation of Cas9. For example, Ferry and colleagues designed a gRNA that forms a hairpin and blocks the spacer domain. The loop of this hairpin is the target of an ASO sequence such that Cas9 activity is dependent on RNase H-mediated cleavage of the loop (Figure 6C) [88].

Another general concept for enzyme-mediated smart NATs uses a nucleic acid input that drives strand-hybridization and -displacement reactions that create substrates for Dicer or Drosha

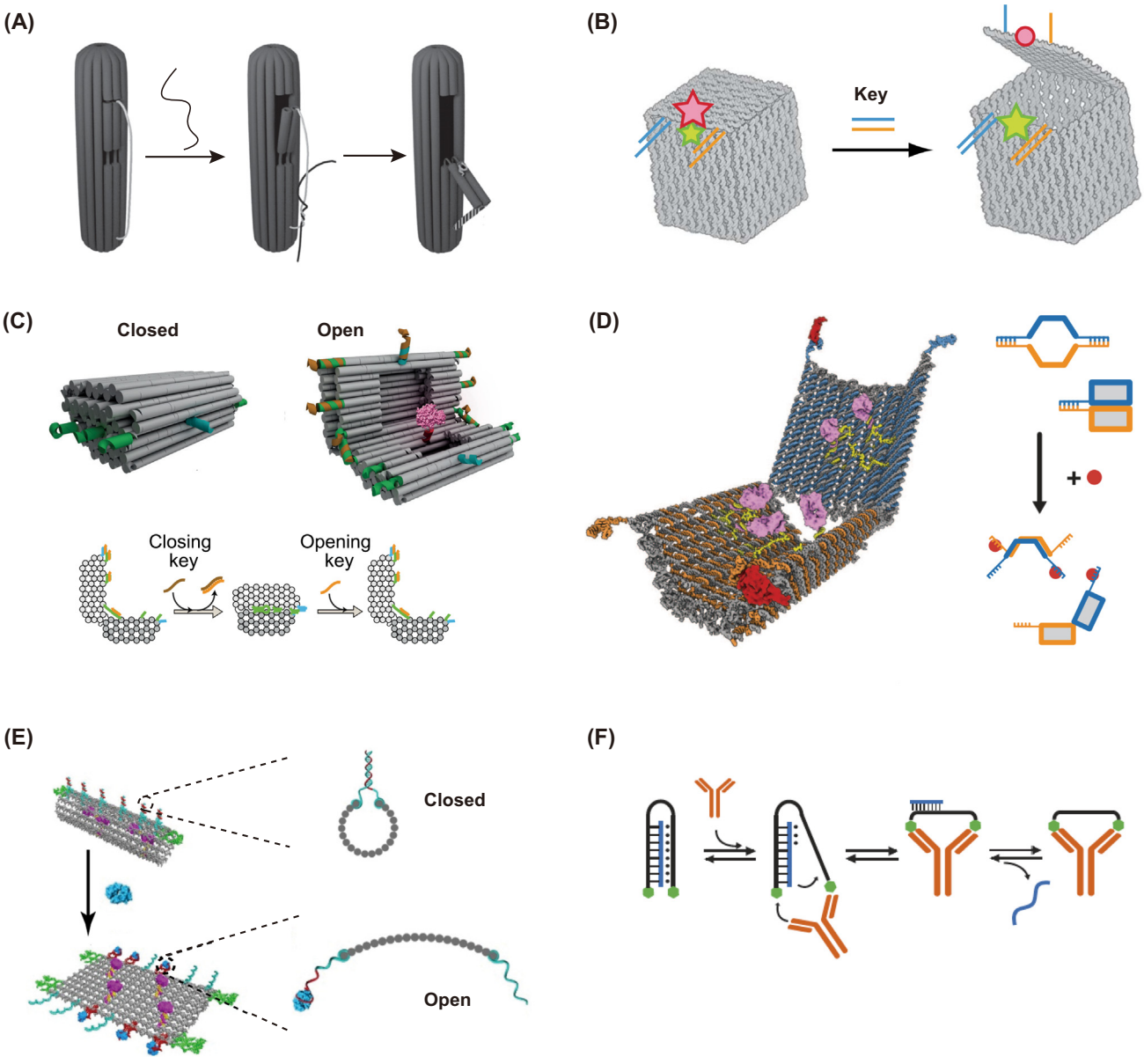

Figure 5. Dynamic DNA Nanostructures for Therapeutics Release Triggered by Hybridization (A), Strand Displacement (B,C), Protein–Aptamer Binding (D,E), and Antibody–Antigen Binding (F). (A) A cylindrical nanorobot with a switchable flap is opened upon hybridization of single-stranded (ss) DNA, thus reducing the length of the linker. Images adapted, with permission, from [81]. (B) A DNA nanobox opens its lid by key oligonucleotides via toehold-mediated displacement with the lock duplex. Image adapted, with permission, from [82]. (C) A DNA vault reversibly opens and closes in the presence of open key and closing key strands. Image adapted, with permission, from [84]. (D) A DNA nanobarrel is opened when the complementary fastener strand of the aptamer is displaced by protein binding. Image adapted, with permission, from [96]. (E) A DNA nanosheet forms a tube by hybridization to an aptamer and its complementary fastener strand. The tube is opened after the trigger protein binds to its aptamer. Image adapted, with permission, from [97]. (F) A triplex complex is disrupted via antibody–antigen binding to release the cargo strand. Image adapted, with permission, from [100].

processing which in turn generates active siRNA. In one example, a Dicer substrate becomes inactive when hybridized to a lock strand. An mRNA trigger removes the lock via toehold-mediated strand displacement, allowing Dicer processing (Figure 6D) [89]. In another design,

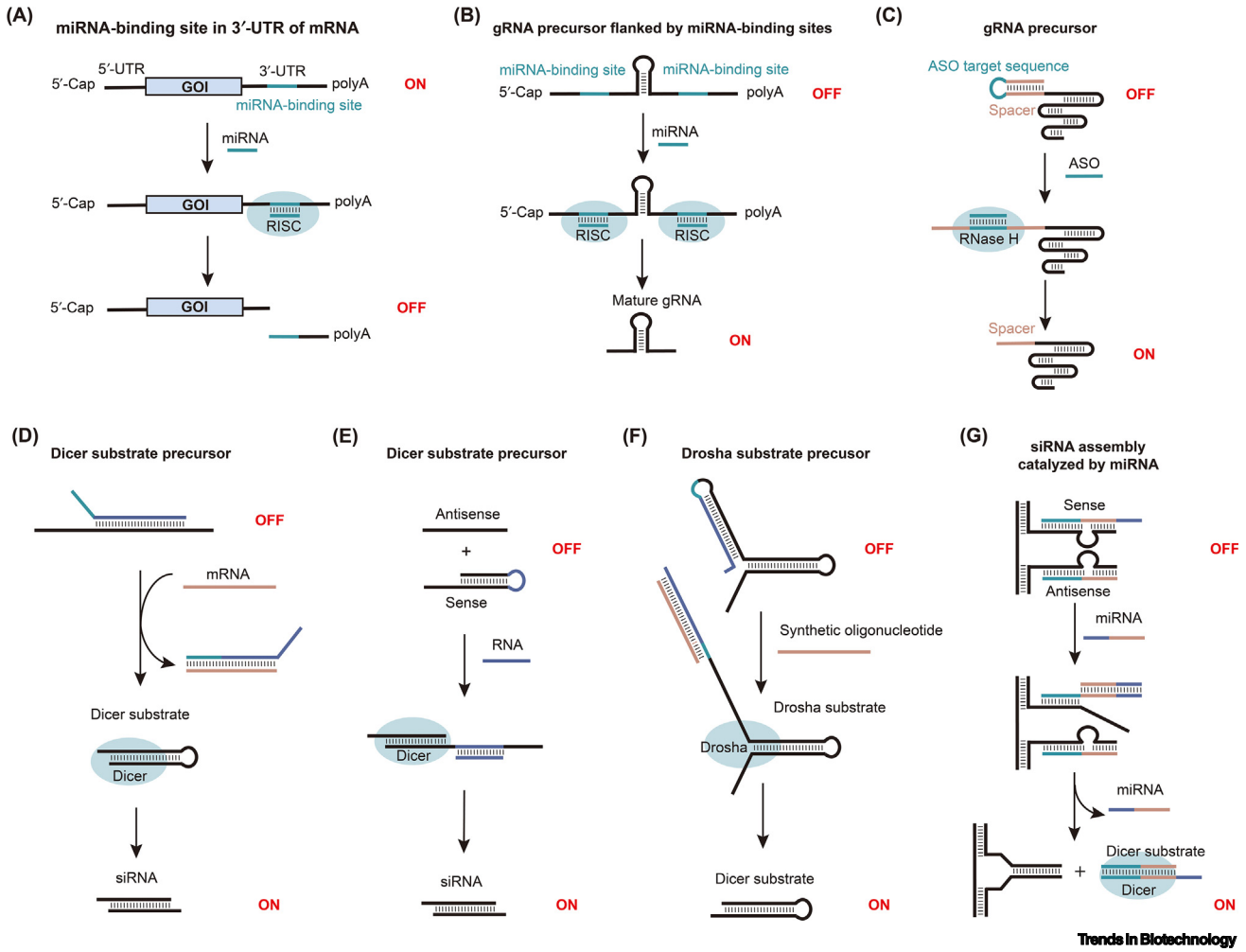

Figure 6. Hybridization- and Strand Displacement-Mediated Enzyme Activation Allows the Generation or Destabilization of Nucleic Acid Therapeutics (NATs). (A) Inserting miRNA target sites into 3′-untranslated region (3′-UTR) of an mRNA for the gene of interest (GOI) enables miRNA- and RNA-induced silencing complex (RISC)-mediated degradation of the mRNA. Image adapted, with permission, from [85]. (B) Guide RNA (gRNA) flanked by miRNA binding sites can be processed by the RISC into mature gRNA after miRNA binding. Image adapted, with permission, from [87]. (C) gRNA with the spacer domain blocked by a stem-loop is activated by RNase H-mediated cleavage after hybridization of an antisense oligonucleotide (ASO) to the loop domain. Image adapted, with permission, from [88]. (D) A linearized Dicer substrate hybridized to a lock strand is activated by an mRNA trigger via toehold-mediated displacement. Image adapted, with permission, from [89]. (E) A hairpin-shaped sense strand is linearized when bound to the trigger RNA, and reacts with the antisense strand, activating Dicer processing. Image adapted, with permission, from [90]. (F) A Drosha substrate precursor is activated by a synthetic oligonucleotide and then processed by Drosha. Image adapted, with permission, from [91]. (G) Split Dicer substrates patterned on a DNA scaffold reassociate and assemble into an siRNA precursor induced by miRNA-triggered cascade reaction. The guide and passenger strands of siRNA are split into two DNA/RNA hybrids that are brought into spatial proximity through hybridization to a DNA scaffold. One of the hybrids possesses a miRNA-complementary toehold: the trigger miRNA induces dissociation of one DNA/RNA duplex and further allows DNA/DNA hybridization, leading to the formation of a siRNA precursor. The released miRNA can further amplify this siRNA signal by triggering the next strand displacement to continuously generate siRNA. Image adapted, with permission, from [95].

an RNA trigger binds to a hairpin-shaped sense strand, allowing hybridization to the antisense strand, which activates Dicer processing of the duplex (Figure 6E) [90]. Similarly, a conditional Drosha substrate can be activated by hybridization to a trigger oligonucleotide to open a hairpin that blocks Drosha binding (Figure 6F) [91]. Although random trigger sequences were used in these studies, these strategies are general and can be adapted to any trigger nucleic acid, including endogenous RNA molecules. These strategies have yet to be demonstrated *in vivo*, likely because

of the challenges regarding the stability and delivery of the pro-drug RNA, and the choice of an endogenous trigger RNA with adequate concentration. To overcome these challenges, Ren and coworkers incorporated an miRNA-triggered cascade reaction with *in situ* RNA assembly [92–94] (Figure 6G) and delivered the system using polyethylenimine [95].

The previously summarized smart NATs with hybridization- and strand-displacement reactions as signal transducers and endogenous transcripts as the input are modular because of the predictability of nucleic acid complementarity. As more disease-related transcripts are discovered by transcriptomics, smart NATs controlled by these specific transcripts can potentially enable on-demand and autonomous therapeutics based on *in situ* diagnosis with reduced off-target effect and toxicity.

Protein-Protein/Aptamer Interactions Induce Dissociation of Nucleic Acid Complexes for the Release of Therapeutics

To this point we have discussed the general concept of using intracellular inputs such as miRNAs, mRNAs, small molecules, and proteins to trigger the activation/deactivation of gene regulation. However, it is also desirable to create drug delivery systems that detect extracellular markers, such as cell-surface receptors that are associated with a disease state. To achieve this goal, 'spring-loaded' DNA nanostructures, also known as 'nanorobots', were engineered to release their payload upon encountering a cell-surface marker (Figure 5D,E). This approach takes advantage of protein–aptamer binding to mediate duplex dehybridization and therapeutic payload release in proximity to the target cell. In this strategy, the lock is usually a DNA duplex that undergoes target-induced switching between an aptamer–complement duplex and an aptamer–target complex. Douglas and coworkers designed a DNA barrel closed by two aptamer locks for different protein targets, which selectively released fluorescent-labeled antibodies when incubated with cells that express the corresponding key proteins (Figure 5D) [96]. In another example, Li and colleagues designed a hollow tube-shaped DNA origami structure assembled from a sheet by hybridizing predesigned fastener strands containing DNA aptamers that bind to nucleolin. Nucleolin is a protein specifically expressed on tumor-associated endothelial cells. Interestingly, aptamer binding to surface-expressed nucleolin leads to both binding of the nanostructure to the cell as well as opening of the origami tube and exposure of thrombin in tumor-associated blood vessels, thereby inducing coagulation and tumor necrosis (Figure 5E) [97]. These smart DNA nanostructure designs can enable drug delivery in a programmable manner in targeted tissue. To this end, the stability of the nanostructure and the activation of a non-specific immune response by these foreign nucleic acids need to be considered. In addition, protein–aptamer-triggered autonomous release of therapeutics from DNA nanostructures relies on the efficiency and yield of the structure-switching element. The response is highly dependent on the tug-of-war between aptamer–target affinity, aptamer–complement affinity, and their respective concentrations; hence creating and testing new triggered structure-switching aptamers is difficult to optimize without extensive experimental testing. For the purpose of autonomous release triggered by disease-associated cell-surface markers, SELEX or **Cell-SELEX** (Box 1) [98] should be performed with the specific marker or cell subtype to generate standard aptamers. Furthermore, these aptamers annealed with an antisense sequence should be screened to select aptamers that switch structures upon target binding [99] (Box 1).

Antibody–antigen binding is another modality that triggers release of cargos, such as antibody-powered nucleic acid release reported by Ranallo and colleagues (Figure 5F) [100]. The clamp-like structure is conjugated at the two ends with antigens. Antibody binding to the antigens induces disruption of Hoogsteen interaction and opening of the triplex complex, resulting in the release of the cargo due to the weak Watson–Crick interaction. This system was demonstrated

in buffer but has not yet demonstrated potential for release of therapeutic oligonucleotides in cell experiments.

Concluding Remarks and Future Perspectives

As summarized previously, a wide range of programmable modalities have been adopted to realize controlled activation or deactivation of NATs. Based on the source of the trigger molecule, smart NATs can be controlled by externally added triggers or endogenously expressed triggers. Photochemical control provides spatiotemporal precision, but its clinical translation as smart therapeutics is limited by the tissue penetration of UV or visible light. Molecular triggering utilizing RNA aptamer-based ribodevices, strand hybridization/displacement, and protein–ligand binding is more suitable for building autonomous agents that respond to disease markers and can be programmed to execute therapeutic functions based on *in situ* diagnosis. However, to this end, the discovery of disease- or cell subtype-specific markers (e.g., metabolites, proteins, transcripts) and the ever-expanding number of molecular devices that interact with them under physiological conditions, are crucial for the growth of this field (see Outstanding Questions). Molecular triggering leveraging strand hybridization/displacement is advantageous because it is modular and the triggering sequence can be arbitrary. Because of the predictability of nucleic acids, computing-aided design has been used to predict the sequences and structures of these devices, such as NUPACK [101], the toehold-switch design web tool [102], and iSBHfold algorithm to optimize ASO-sensing loops in the gRNA precursor design shown in Figure 6C [88]. In addition, robust reporter cell lines make high-throughput, quantitative screening and characterization of large library of molecular devices possible.

Although proof-of-concept of smart nucleic acids has been extensively demonstrated, currently no smart NAT has reached the clinic. One of the hurdles is the limited number of approved (conventional) nucleic acid therapeutics. So far, only a small group of NATs have been approved, and these are mostly aimed at treating orphan and/or severe disease. However, more therapeutic candidates for a larger range of diseases are in the pipeline and will soon come online [103]. Furthermore, in addition to the robustness of the triggering mechanism, clinical translation of smart NATs also requires the programmable modality or the signal transducer to recognize trigger molecules under physiological conditions with both high sensitivity and specificity. This is because of the low concentration of disease-associated biomolecules and the crowded biological environment in which they exist. Therefore, improving the sensitivity and specificity of smart NATs will be a main focus in this field. Upon overcoming these two hurdles, smart NATs will reach the clinic in the near future, and especially for the treatment of cancer, inflammatory diseases, and neurodegenerative disorders which often involve elevated levels of disease biomarkers such as cytokines, as well as disease-specific transcripts and proteins. These biomarkers can offer appropriate input signals to control therapeutic activities.

One direction toward autonomous functionality of NATs is the development of 'on-demand' therapeutics, which are reversible and controlled in an analogous manner by implementing continuous computation in response to changing disease-indicating inputs. This will require increasing the lifetime of the smart NATs by incorporating chemical modifications. Another aspect that needs to be considered for clinical translation of smart NATs is their cellular entry. So far, the most commonly used vehicles for mRNA therapeutics and oligonucleotide therapeutics are LNPs. Inorganic nanoparticles such as gold nanoparticles have also been demonstrated to deliver smart NATs [79].

In addition to the previously-mentioned major mechanisms for controlling the activity of smart NATs, other mechanisms have also been reported in the literature, such as chemical activation

Outstanding Questions

How can the sensitivity and specificity of smart NATs be improved such that they can be induced by ultra-low concentrations of trigger molecules with minimal leakage?

Is it possible to design and predict smart NATs *in silico* to reduce the time and effort spent in screening and testing?

Could smart NATs be combined with specific tissue-targeting nanomaterials to further enhance the specificity of the therapeutics?

Is it possible to create NATs that are continuously present *in vivo* and that autonomously 'diagnose' and treat disease states based on continuous computation of the ever-changing molecular profiles of the cell?

[104] and enzyme (Csy4) activation of gRNA [88]. Some novel mechanisms are also under development, such as mechanical force-triggered release of therapeutics [105]. DNA nanostructures have been used to study molecular mechanics in cells by leveraging force-induced melting of DNA hairpins, duplexes, and nanostructures [106,107]. Although their applications are focused on probing force by a fluorescence-based readout, mechanical force-triggered release of therapeutics, including intercalating molecules, oligonucleotides, and proteins, from nucleic acid nanostructures may be around the corner.

Declaration of Interests

The authors declare no conflicts of interest.

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